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CD4$^+$ T Cells Are Ineffective in Clearing a Pulmonary Infection

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Recovery from influenza virus infection is dependent on T cell functions which can be provided either by CD8 or CD4 T cells. To identify the functions involved in recovery promoted by CD4 T cells, we have studied the course of the infection in B-cell deficient μMT mice which had been depleted of CD8 T cells by antibody treatment. Upon infection with PR8 [A/PR/8/34(H1N1)], such B- and CD8 T cell-deficient mice mounted strong CD4 T cell responses that were comparable in size and cytokine secretion to those seen in intact mice. Yet, these B- and CD8 T cell-deficient mice could not clear the infection, in contrast to (CD8-depleted) mice containing both B- and CD4 T cells. These findings indicate that the promotion of the T-dependent antibody response is an indispensable component in the CD4 T cell-dependent recovery process.

INTRODUCTION

In mammals, influenza type A virus typically produces a cytopathic infection of the epithelium that covers the airways of both the upper and the lower respiratory tract (RT). The infection in mice has been used extensively in the past to study host defense mechanisms that operate at these sites of the RT. It is now firmly established that recovery from this infection is dependent on T cell functions, i.e., does not occur in athymic nude or experimentally T cell-depleted mice (Wells *et al.,* 1981; Doherty *et al.,* 1992). More recent studies revealed that both CD8 and CD4 T cells are capable of promoting recovery (Doherty *et al.,* 1992), apparently through distinct mechanisms, as CD8 T cells required a MHC-restricted interaction with infected epithelial cells (Hou and Doherty, 1995), while this was not the case for CD4 T cells (Topham *et al.,* 1996a). The latter observation left open the possibilities that CD4 T cells promoted clearance by killing infected epithelial cells in a non-MHC-restricted fashion such as Fas/Fas-ligand interaction, activation of innate defense mechanisms such as macrophages, neutrophils, and NK cells, release of cytokines with antiviral activities, and/or promotion of a T-dependent anti-viral antibody response.

We have previously shown that infected athymic nude, but not T and B cell-deficient SCID, mice could be cured by adoptive transfer of virus-specific CD4 T cell clones (Scherle *et al.,* 1992) and that infected SCID mice could be cured by treatment with anti-viral antibody (Palladino *et al.,* 1995). These observations are consistent with the idea that CD4 T cells promote recovery through promotion of an antibody response. The problem, however, was to verify that the local antibody concentrations in the RT required for virus clearance in the passive treatment protocol could actually be achieved by the active antibody response. Furthermore, the failure of individual CD4 T cell clones to effect clearance did not exclude the possibility that the endogenous polyspecific CD4 T cell response would be effective. The CD4 T cells clones, though all of type 1, may not have secreted the most effective combination of cytokines or may not have migrated properly *in vivo* to the site of infection. It was important, therefore, to assess the efficacy of the active polyclonal CD4 T cell response on its own in the recovery process. This was done by studying the course of the viral infection in μMT mice (Kitamura *et al.,* 1991) which are B cell-deficient because of a disrupted membrane IgM gene and which were additionally depleted of CD8 T cells by treatment with CD8-specific mAb. These experiments showed that the endogenous active CD4 T cell response on its own (in conjunction with innate defense mechanisms) is incapable of resolving a pulmonary infection with the influenza type A virus PR8.

MATERIAL AND METHODS

Mice

BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME) or Harlan Sprague Dawley (Indianapolis, IN). Two homozygous male μMT mice were kindly provided by the Institute for Genetics (Cologne, FRG) through L. Shultz (Jackson Laboratory). μMT mice are B cell deficient because of disruption of the gene

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coding for membrane IgM (Kitamura *et al.,* 1991). They were backcrossed to BALB/c. After the sixth backcross, a line homozygous for the μMT disruption was generated by intercrossing (termed BALB/c-μMT). Mice were maintained in microisolators under specific pathogen-free conditions.

Viruses

Influenza type A virus strain A/PR/8/34 (H1N1) (PR8) was originally obtained from Mt. Sinai Hospital (New York). The infectious stock used in this study was grown in the allantoic cavity of embryonated hen's eggs and contained $10^{8.4}$ $TCID_{50}$ (50% tissue culture infectious dose for Madin Darby canine kidney (MDCK) cells) and $10^{7.7}$ MID_{50} (50% mouse infectious dose) per ml. The infectious influenza type B virus stock B/Lee/40 (Lee) contained $10^{7.8}$ $TCID_{50}$/ml. Purified and UV inactivated virus was prepared as described (Mozdzanowska *et al.,* 1997).

Media and solutions

Iscove's modified Dulbecco's medium (Gibco), supplemented with 0.05 mM 2-mercaptoethanol, 0.005 mg/ml transferrin (Sigma), 2 mM glutamine (JRH Biosciences, KS), and 0.05 mg/ml gentamicin sulfate (Whittaker, MD) (Isc-CM) was used and further supplemented, as indicated, with fetal calf serum (FCS) (HyClone), bovine serum albumin (BSA) (Sigma), or 5-bromo-2-deoxy-uridine (BudR, Sigma). Phosphate buffered saline containing 0.04% NaN_3 (PBSN) was supplemented, as indicated, with BSA.

Cell lines and antibodies

MDCK and A20 were maintained in Isc-CM–5% FCS. P1.HTR (Woegel *et al.,* 1987), a cell line derived from the P815 mastocytoma line, was maintained in Isc-CM-BudR (0.1 mg/ml). DA-1, an IL-3 and GM-CSF-responsive myeloid cell line, was maintained in Isc-CM–FCS (5%) supplemented with IL-3 (250 U/ml). IL-3, IL-4, and IFN-γ secreting cell lines had been obtained from Karasuyama and Melchers (Karasuyama and Melchers, 1988). The following hybridoma antibodies were used: R1.3-12 (rat anti-mouse IgG2b), 53-6.72 (rat anti-mouse CD8, ATCC TIB 105), GK1.5 (rat anti-mouse CD4, ATCC TIB 207), M1/9.3.4.HL.2 (rat anti-mouse CD45, ATCC TIB 122), 187.1 (rat anti-mouse c_κ, ATCC HB 58), RA3-3A1/6.1 (CD45R-B220, ATCC TIB 146). Rt2-8 is a murine hybridoma specific for a determinant shared by all rat mAbs included in this analysis. The precise nature of this shared determinant is not known. Purified Rt2-8 was labeled with FITC by means of the FluoroTag FITC conjugation kit (Sigma, St. Louis, MO) according to the protocol recommended by the manufacturer.

CD8 T cell-depletion and infection of mice

Mice (8- to 12-week-old) were depleted of CD8 T cells by repetitive (5- to 6-day interval) intraperitoneal (i.p.) injections of purified CD8-specific mAb 53-6.72 in 0.2 ml phosphate-buffered saline (PBS), the first injection being 200 μg and all subsequent injections 100 μg. Control mice received an analogous treatment with purified rat-Ig or the isotype-matched (IgG2a) rat mAb R1.3-12. Three days after initiation of the CD8 depletion, anesthetized mice (i.p. injection of 0.2 ml ketamine [10 mg/ml]/xylazine [2 mg/ml]) were infected by intranasal inhalation of 30 μl PR8 ($\sim$100 $TCID_{50}$). Mice were euthanized by i.p. injection of ketamine/xylazine and subsequent exsanguination by heart puncture. Lung, lymph nodes, and spleen were harvested. One lung lobe was immediately frozen for subsequent determination of virus titer, which was done as described (Palladino *et al.,* 1995), and one lobe was used for isolation of lung parenchymal cells for measurement of MHC class I-restricted cytotoxic activity. Cells were isolated from lung and lymphoid tissues as described (Liang *et al.,* 1994).

FCM analysis

The FCM staining procedure was as follows: 25 μl of cells (0.5×10^6) plus 50 μl of MAb (hybridoma culture supernatant) were incubated in microfuge tubes on ice for 45 min, washed once, resuspended in 50 μl of an optimal dilution of FITC-labeled mouse anti-rat Ig mAb Rt2-8, incubated on ice for 45 min, washed once, and resuspended in 0.2 ml PBSN-1% BSA, which was the diluent and wash fluid used in these assays. Samples were analyzed with an Ortho Cytofluorograf System 50, connected to a Model 2150 data handling system (Ortho Diagnostic System, Inc., MA). The gating was set by forward and right-angle scatter for viable lymphocytes and 2000 events were analyzed.

Analysis of CD8 T cell cytotoxic activity

Target cells were prepared as follows: P1.HTR cells were resuspended at 2×10^6 cells/ml in Isc-CM (without serum). Virus (PR8 or B/Lee)-containing allantoic fluid ($\sim 10^{6.5}$ $TCID_{50}/10^6$ cells) was added and the cells incubated for 1 h at 37° with occasional rocking. The culture was diluted fivefold with Isc-CM–5% FCS and incubated overnight ($\sim$16 h) at 32°. Aliquots (8 ml) of the culture were transferred to 15-ml centrifuge tubes. One hundred percent Percoll (Pharmacia) was added to give a final concentration of 33%, the suspension underlayed with $\sim$2 ml of 70% Percoll and spun for 10 min at 600 *g* at room temperature. The cells at the 33%/70% interface were harvested, diluted in Isc-CM–FCS (5%), pelleted, and resuspended at 10^7/ml in the above medium. The desired number of cells was incubated for 45 min at 37° with ^{51}Cr (0.1 μCi/10^6 cells, ICN), washed, and dispensed at 10^4 cells per well into round-bottom microtiter plates. Lung parenchymal cells were prepared as described and dispensed into the plate to give the desired effector:target (E:T) ratios. The plates were briefly spun to deposit

the cells into the bottom of the wells and the amount of ^{51}Cr released into 100 μl of medium (1/2 of total culture volume) was determined after 4 h of incubation at 37°. The percentage of specific release was computed as described (Scherle *et al.,* 1992).

Analysis of CD4 T cell activities

CD4 T cell activity was assessed by measuring cytokine secretion in 0.2-ml restimulation cultures set up in 96-well flat-bottom microtiter plates. The medium was Isc-CM (without serum). Stimulator cells were prepared as follows: A20 cells in Isc-CM were incubated for 1 h at 37° with purified and UV inactivated virus, ~350 ng/10^6 cells. The suspension was then irradiated (4400 rad) and the cells pelleted, resuspended in Isc-CM, and dispensed into microtiter plates to give 10^5 cells/well. Responder cells isolated from mediastinal lymph nodes (MedLN) and spleen were then added at various responder cell concentrations (10^4 to 10^6/well, several replicates per responder cell concentration) and the cultures (0.2 ml total volume) incubated for 24 h at 37°. Cell-free culture medium was then harvested for measurement of cytokine concentration.

IL-3/GM-CSF was measured by means of the DA-1 bioassay. Aliquots (50 μl) of DA-1 cells (4×10^5/ml Isc-CM−FCS (2%)) were added to 50 μl of test sample dilutions, usually in four replicates, in flat-bottom 96-well microtiter plates. The plates were incubated for 2 days and the concentration of viable DA-1 cells determined by MTT assay as described (Haberman *et al.,* 1990). Each assay was standardized by a parallel titration of a sample of known IL-3 concentration. The IL-3/GM-CSF concentration is expressed in units/ml (1 U ~ 10 pg).

IL-4 concentration was measured by capture ELISA using plate-bound mAb 11B11 as capture reagent and biotinylated anti-IL-4 mAb BVD6-24G2 (PharMingen) as measuring reagent. The assay was developed by sequential incubation with Streptavidine-AP (Sigma) and pNPP (Sigma) and optical density read in an ELISA reader (Molecular Devices) at $A_{405-750}$. The threshold sensitivity of the assay was ~50 pg/ml.

IFN-γ concentration was measured by capture ELISA using plate-bound R4-6A2 mAb for capture and biotinylated mAb XMG1.2 (PharMingen) as detecting reagent. The assay was developed and read as above. The threshold sensitivity was ~200 pg/ml.

RESULTS

B cell-deficient μMT mice cannot recover from a pulmonary infection with PR8 if they are depleted of CD8 T cells

Three challenge experiments were performed. Experiment 1 was performed with mice generated by brother–sister matings after the second backcross of the μMT strain to the B10D2 mouse strain. In this experiment, CD8-depleted and control μMT (−/−) mice were compared to CD8-depleted and control μMT (+/−) and (+/+) littermates. Experiments 2 and 3 were performed with a BALB/c-μ MT (−/−) mouse line that was generated after the sixth backcross to BALB/c. In all experiments, mice received a total RT infection with 15–45 MID_{50} of PR8 and the status of the infection was determined 7, 11, and 17 or 18 days later.

Table 1 shows that μMT mice contained, as expected (Kitamura *et al.,* 1991), only few B cells ($B220^+$) in secondary lymphoid tissues and, as reported (Kitamura *et al.,* 1991), the few $B220^+$ cells detected expressed this antigen at decreased density compared to B cells from normal mice (data not shown). Although these mice have, on average, greatly reduced B cell function, the defect shows some leakiness, which manifested itself most prominently in readily detectable though still subnormal concentrations of IgG2a and IgG2b in serum (our unpublished observations). In spite of this leakiness, the μMT mice described in these studies failed to mount anti-viral antibody responses that exceeded 0.2% of the response of intact mice (data not shown). However, unequivocal responses, some amounting to more than 1% of the normal response, were sporadically observed at later time points (our unpublished observations). Table 1 also demonstrates that the CD8 T cell-depletion protocol used here resulted in strong and sustained depletion of CD8 T cells. It is of note also that only insignificant numbers (<2%) of cells with bound treatment mAb were detectable in these mice at both time points (data not shown). Effective depletion of CD8 T cells was demonstrated also by measuring cytotoxic activity exhibited by the pulmonary cellular infiltrates (Fig. 1). Although low cytotoxic activity was detectable in μMT (-CD8) mice 7 days after infection, it was significantly smaller than in μMT (control) and BALB/c mice and had disappeared by day 11. It is noteworthy also that μMT (control) and BALB/c mice developed quite similar CTL activity in their lungs, indicating that B cells are not important for induction of influenza virus-specific CD8 T cell responses (Topham *et al.,* 1996a).

Table 2 shows the course of the infection in these mice. It is evident, first, that 90% of B- and CD8 T-cell containing mice, (+/+) and (+/−) littermates in experiment 1 and BALB/c mice experiments 2 and 3, had cleared the infection by day 11. Virus clearance appeared to be somewhat delayed in CD8- or B cell-deficient mice, which is in general agreement with previous reports (Bender *et al.,* 1992; Graham and Braciale, 1996; Doherty *et al.,* 1997). Importantly, however, none of 20 BALB/c-μMT (-CD8) mice managed to clear the infection; all contained high virus titers in their lungs on day 11, showed a high rate of mortality, particularly between 10 and 18 days after infection, and, if they survived until day 17 to 18, showed further increases in the titer of virus in their

TABLE 1

Lymphocyte Composition in Experimental Mice

		Percentage of cells					
		CD4+		CD8+		B220+	
Donor mouse	Tissue	Day 7	Day 11	Day 7	Day 11	Day 7	Day 11
BALB/c	MedLN	36	28	15	9	32	37
	Spleen	23	18	11	9	49	36
BALB/c(−CD8)	MedLN	nd	45	nd	1	nd	26
BALB/c-μMT	MedLN	70	nd	28	nd	1	nd
	Spleen	34	28	16	13	3	5
BALB/c-μMT(−CD8)	MedLN	82	85	3	0	1	1
	Spleen	45	52	1	0	2	4

Note. MedLN and spleens were obtained 7 or 11 days after total RT infection with ~10 MID_{50} of PR8. The tissues of four to five mice within each group were pooled prior to isolation of cells. FCM analysis was performed as indicated under Materials and Method, using GK1.5 and 536.72 for detection of CD4 and CD8 T cells, respectively, and RA3-3A1 for detection of B220 positive cells. Rat mAb was detected with FITC-labeled mAb Rt2-8. Cell recovery from these mice is shown in Table 3.

lungs. Taken together, these observations indicate that B cells make an important and perhaps indispensable contribution in recovery of CD8 T cell-deficient mice and that CD4 T cells are incapable of resolving this infection in conjunction with innate defense mechanism. An analogous study by Topham and Doherty (1997) in which recovery from the less pathogenic X31 virus infection was tested, provided similar data in that only ~10% of CD8-depleted μMT mice managed to clear the infection.

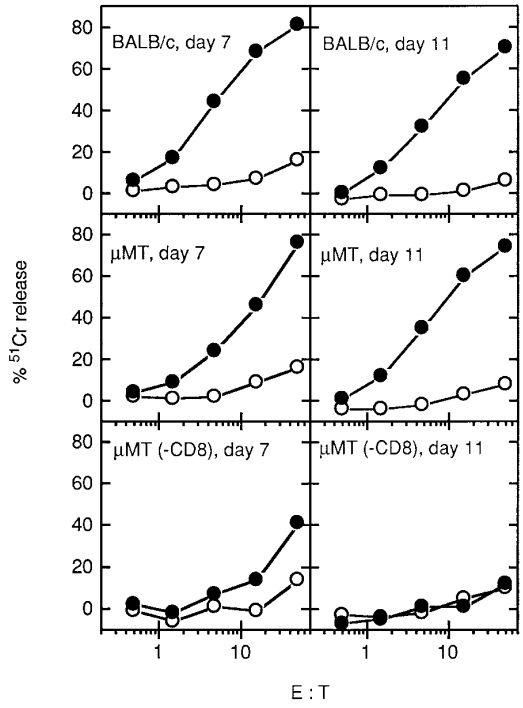

FIG. 1. MHC class I-restricted cytotoxic T cell activity displayed by lung parenchymal cells. Effector cells were isolated by collagenase treatment of lung tissue 7 and 11 days after infection with PR8. The cells were tested at the indicated E:T ratios (relative to all viable cells) in a 4-h ^{51}Cr-release assay against PR8 (filled dots)- and Lee-infected (open dots) P1.HTR target cells. Lee is a influenza virus strain of type B which is immunologically non-cross-reactive with the type A strain PR8. Since P1.HTR target cells are MHC class II negative, only MHC class I-restricted T cell activity is measured in this assay. Each dot shows the mean specific ^{51}Cr release from four replicate cultures.

The inability of CD4$^+$ T cells to control the infection is not due to their ineffective stimulation in the absence of B cells

As CD4 T cell responses to some antigens have been reported to be reduced in B cell deficient mice (HayGlass *et al.,* 1986; Janeway *et al.,* 1987; Ron and Sprent, 1987; Kurt-Jones *et al.,* 1988; Liu *et al.,* 1995; Constant *et al.,* 1995; Baird and Parker, 1996; Vella *et al.,* 1996), it was important to assess the size of the CD4 T cell response in PR8-infected μMT (-CD8) mice. Table 3 shows that very similar numbers of CD4 T cells were recovered from lungs and MedLN of BALB/c and BALB/c-μMT mice. To show that these cellular CD4 T cell responses also comprised comparable numbers of virus specific cells, various doses of these cells were restimulated *in vitro* with irradiated A20 cells that had been pulsed with a relatively low dose of UV inactivated PR8 virus. Given that processing of inactivated virus through the endocytic pathway leads to preferential presentation of viral determinants by MHC class II proteins (Morrison *et al.,* 1986; Germain *et al.,* 1996), these culture conditions would be expected to restimulate preferentially virus-specific CD4 T cells. CD4 T cell activation was assessed by measuring the concentration of IL-3/GM-CSF released into the culture media after one day of incubation. Figure 2 shows that the cell populations recovered from the various groups of mice 7 and 11 days after infection responded comparably in this assay, thus supporting the notion that these mice generated virus-specific CD4 T cell responses of comparable strength.

TABLE 2

CD8-Depleted μMT Mice Cannot Clear the Pulmonary Infection

Experiment	Group	Day after infection	Cured/tested	Lung virus titer $\log_{10}$ (mean ± SEM (*n*))	Cumulative mortality (%)
1	Lm (+*l*)	11	3/3	0	0
	Lm (+*l*) (−CD8)	11	2/2	0	0
	μMT	11	2/3	4.1 (1)	0
	μMT (−CD8)	11	0/3	5.1 ± 0.4 (3)	0
	μMT (−CD8)	18	0/4	5.7 (1)	75
2	BALB/c	11	4/4	0	0
	BALB/c-μMT	11	0/4	1.9 ± 0.3 (4)	0
	BALB/c-μMT (−CD8)	11	0/4	5.6 ± 0.2 (4)	0
	BALB/c-μMT (−CD8)	17	0/4	7.0 ± 0.8 (2)	50
3	BALB/c	11	3/4	1.6	0
	BALB/c (−CD8)	11	1/5	1.5 ± 0.1 (4)	0
	BALB/c-μMT (−CD8)	11	0/5	6.6 ± 0.1 (3)	40

Note. The mice used in experiment 1 are from brother/sister matings after the second backcross of μMT mice to the B10D2 strain and in experiments 2 and 3, mice obtained after the sixth backcross of μMT to the BALB/c strain. Mice were depleted of CD8 T cells (−CD8) by treatment with rat anti-CD8 mAb 536.72 3 days before and 1, 6, and 12 days after total RT infection with 15 MID_{50} (experiments 1 and 2) or 45 MID_{50} (experiment 3) of PR8. Virus titer in lung extracts was determined at the indicated time points. Failure to detect virus after inoculation of undiluted lung extract (2×50 μl) into the allanoic cavity of embryonated chicken eggs was considered to indicate complete clearance of the infection.

As CD4 T cells of type 1 have been reported to be more effective in promoting recovery than CD4 T cells of type 2 (Graham *et al.,* 1994; Moran *et al.,* 1996), we also tested whether the presence or the absence of B cells may have altered the type of the response. As shown in Table 4, MedLN and lung cells from all groups of mice released comparable amounts of IFN-γ and no detectable IL-4 upon restimulation with PR8-pulsed A20 cells *in vitro,* thus providing no evidence for a significant change in type. In sum, BALB/c-μMT (-CD8) mice appeared to have generated CD4 T cell responses that were equivalent in size and type to the ones generated in BALB/c and μMT mice. It therefore appears that even strong CD4 T cell responses of type 1 are ineffective, on their own, in promoting recovery from this infection.

TABLE 3

Total Cell Recovery from MedLN and Lungs of Infected Mice

	Average cell recovery ($\times 10^{-6}$) per mouse 7 days after infection			
	Med LN		Lung	
Donor mouse	Total	CD4	Total	CD4
BALB/c	9.9	3.5	5.5	0.7
BALB/c-μMT	4.9	3.4	4.2	0.5
BALB/c-μMT(−CD8)	5.3	4.3	3.9	0.5

Note. The cell recovery data were obtained from two independent experiments, each comprising five and three mice per group, respectively. Within each group, MedLN or lungs were pooled prior to isolation of cells. Total indicates the number of viable cells (trypan blue negative) recovered per mouse. The number of $CD4^+$ cells was computed on the basis of the FCM analysis (Table 1) performed on each cell preparation.

DISCUSSION

Previous studies have established that mice which lack CD8 T cells can recover from an influenza type A virus infection (Eichelberger *et al.,* 1991; Bender *et al.,* 1992; Scherle *et al.,* 1992), though less effectively than immunologically intact mice (Bender *et al.,* 1992). This study was conducted to identify the mechanism(s) by which the infection is being resolved in this situation. There are several conceivable mechanism(s) that could operate here, including (1) contact-dependent killing of infected cells by cytotoxic CD4 effectors (Hou *et al.,* 1993), (2) inhibition of virus replication by release of cytokines with anti-viral activities such as IFN-γ and TNF-α (Ramsay *et al.,* 1993), (3) recruitment and activation of components of the innate defense system such as NK cells and macrophages (Doherty *et al.,* 1992), and (4) promotion of the T-dependent antibody response (Scherle *et al.,* 1992). There is not much current information about this issue, except for the demonstration that CD4 T cells can promote clearance of the infection in MHC class II-negative chimeric hosts (Topham *et al.,* 1996b), which shows that an MHC restricted recognition of infected epithelial cells by CD4 effector T cells is not required for recovery and is in contrast to virus clearance mediated by CD8 T cells (Hou and Doherty, 1995; Doherty *et al.,* 1997). Another study pointed to the contribution of CD4 T cells to clearance via cytokine secretion by showing that virus clearance was delayed by 3–4 days, though not prevented, if CD8-deficient mice were treated with anti-IFN-γ antibody (Sarawar *et al.,* 1994); CD4 T cells are an important producer of IFN-γ in this situation (Doherty *et al.,* 1992; Baumgarth *et al.,* 1994). The present study further clarifies the question of how CD4 T cells

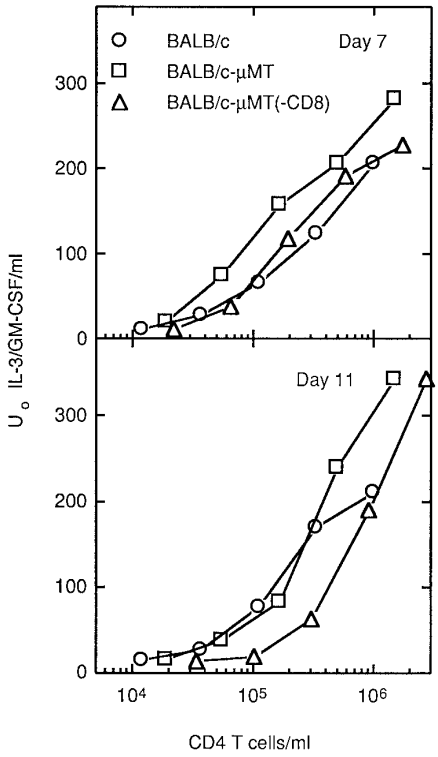

FIG. 2. IL-3/GM-CSF secretion by MedLN after restimulation *in vitro.* MedLN cells, obtained 7 or 11 days after infection, were cultured at various doses with A20 cells pulsed with UV-inactivated PR8. Responder cell doses give the number of CD4 T cells per milliliter of culture, as determined by FCM analysis of the various cell preparations. Each culture contained 0.2 ml. The responders are from BALB/c (circles), BALB/c-μMT (squares), and BALB/c-μMT (-CD8) (triangles) mice. The concentration of IL-3/GM-CSF in culture media was determined by the DA-1 bioassay after 24 h of incubation.

promote clearance in the absence of CD8 T cells by showing that an interaction between CD4 T and B cells is an indispensable component of the CD4-dependent recovery process.

The CD4 T–B cell interaction might be required for two reasons. (1) B cells may be needed for optimal activation of the CD4 T cell response *in vivo.* Prior evidence on this issue is ambiguous in that examples of both diminished (Ron and Sprent, 1976; HayGlass *et al.,* 1986; Janeway *et al.,* 1987; Kurt-Jones *et al.,* 1988; Constant *et al.,* 1995; Liu *et al.,* 1995; Baird and Parker, 1996; Vella *et al.,* 1996) and unaltered (Sunshine *et al.,* 1991; Epstein *et al.,* 1995; Phillips *et al.,* 1996; Topham *et al.,* 1996a) CD4 T cell responses in B cell-deficient mice have been reported. We found that the CD4 T cell response to influenza virus infection was not measurably diminished in B cell-deficient mice (Table 2; Fig. 1), which is in agreement with the study of Topham *et al.* (1996a). Another possible consequence of a change in stimulator cell population could be the deviation of the CD4 T response toward a distinct cytokine secretion profile (Fitch *et al.,* 1993). Current evidence indicates that CD4 T cell responses of type 2 (Th2) are less effective in promoting recovery than Th1 (Graham *et al.,* 1994; Moran *et al.,* 1996) and may even be harmful (Graham *et al.,* 1994). However, since B cells have been reported to induce preferentially Th2 responses (Fitch *et al.,* 1993; Saoudi *et al.,* 1995; Taylor-Robinson and Phillips, 1996; Stockinger *et al.,* 1996; Macaulay *et al.,* 1997), their absence would be expected to direct the response toward the more effective Th1, which dominates already the response of intact mice (Carding *et al.,* 1993). It is not surprising, therefore, that the response of B cell-deficient mice was not measurably different in type from the response of intact mice (Table 3). Thus, influenza virus infection induced an effective CD4 T cell response in the absence of B cells. This leaves the induction of a T-dependent anti-viral antibody response as the likely explanation for the requirement of B cells in the CD4-dependent recovery process. Consistent with this conclusion is our previous finding that infected SCID mice can be cured by treatment with anti-viral antibodies (Palladino *et al.,* 1995; Mozdzanowska *et al.,* 1997).

The severity of the virus challenge is obviously an important factor when one tries to define the efficacy of

TABLE 4

Cytokine Secretion by MedLN and Lung Cells

		IFN-γ (ng/ml)		IL-4 (ng/ml)	
Responder cells	Stimulator cells	MedLN	Lung	MedLN	Lung
BALB/c	A20	0.2 (0.1)	0.8 (2.1)	$<$0.05	$<$0.05
BALB/c	A20-PR8	6.1 (4.2)	4.2 (33.0)	$<$0.05	$<$0.05
BALB/c-μMT	A20-PR8	4.3 (1.5)	4.0 (33.6)	$<$0.05	$<$0.05
BALB/c-μMT ($-$CD8)	A20-PR8	6.1 (1.9)	5.5 (42.9)	$<$0.05	$<$0.05

Note. MedLN and lung cells obtained 7 days after infection from the indicated groups of mice were cultured *in vitro* (4×10^6 cells/ml in the case of MedLN and 3×10^6/ml in the case of lung cells) with PR8-pulsed or nonpulsed A20 cells and IFN-γ and IL-4 concentrations in the culture media were determined by ELISA after 24 h of incubation. The data are the means of two to three independent experiments. The data in parentheses are computed values that take into account different frequencies of CD4 T cells in the responder cell preparations (see Tables 1 and 3) and are expressed relative to cultures containing 10^6 CD4 T cells/ml.

host defense systems: the more severe the challenge the higher the demands on the host's defenses. The PR8 virus strain is quite pathogenic for mice and the challenge dose used here (15–45 MID_{50}) represents approximately 0.1 LD_{50} for immunologically intact mice. However, a poor therapeutic efficacy of CD4 T cells on their own was also seen in infections with the much less pathogenic X31 virus strain (Topham and Doherty, 1997). Thus, the low therapeutic efficacy of CD4 T cells in influenza infection appears to be a general phenomenon.

The low therapeutic efficacy of CD4 effector T cells is surprising in view of the fact that the infection induces a strong CD4 T cell response which is characterized by substantial production of IFN-γ and TNF-α/β (Table 3, Carding *et al.,* 1993; Hou *et al.,* 1993). These cytokines exhibit powerful antiviral activities if they are targeted to infected cells (Ramsay *et al.,* 1993). The importance of MHC-mediated targeted release of cytokines by effector T cells has been demonstrated also by Kundig *et al.* (1993). In rats, and presumably also in mice, the epithelia of the RT do not express MHC class II protein under normal conditions (except for epithelium overlaying BALT and type II pneumocytes of alveoli) but class II proteins become induced under inflammatory conditions (IFN-γ) (Steininger and Sickel, 1991). Thus, conditions for MHC-mediated targeted release of cytokines by CD4 effector T cells should be fulfilled during infection.

Analogous approaches as described here have been used to define the therapeutic activities of CD4 T cells in rotavirus and cytomegalovirus infection. Franco and Greenberg (1995) reported that J_HD mice, a mouse strain rendered B cell-deficient because of targeted deletion of the J region of the Ig heavy chain gene (Chen *et al.,* 1993), could not clear a rotavirus infection if they were depleted of CD8 T cells. McNeal *et al.* (1995) tested both J_HD and μMT mice and observed minor differences between these strains in that CD8-depleted J_HD mice could not clear the infection while CD8-depleted μMT mice could, though very slowly. Thus, the findings in rotavirus and influenza virus infection are overall quite similar. By contrast, CD4 T cells appear to be capable of controlling infection of salivary glands with cytomegalovirus, though not of other tissues such as lung and spleen (Jonjic *et al.,* 1994).

The finding that the primary CD4 T cell response cannot promote recovery from infection in the absence of B cells does not mean that CD4 T cells operate solely through promotion of the T-dependent antibody response. Other CD4-dependent activities are likely to contribute but are not sufficiently effective on their own for virus clearance. This is supported by the above-mentioned study of Sarawar *et al.* (1994), showing that treatment of β2m-KO mice with anti-IFN-γ antibody delayed virus clearance by 3–4 days. Also, adoptive transfer of CD4 T cells into infected mice has been reported to modify the course of disease, sometimes beneficially and in other cases harmfully (Leung and Ada, 1982; Taylor *et al.,* 1990; Scherle *et al.,* 1992; Graham *et al.,* 1994). However, since most of these studies were performed with at least partially immunocompetent recipients, they did not reveal how much the transferred CD4 T cells contributed directly to the final virus clearance. Immunodeficient recipients have been used in only few studies. Lightman *et al.* (1987) transferred purified CD4 memory T cell populations into lethally irradiated recipient mice and reported than one cell preparation cured the infection while a second and apparently purer preparation was ineffective. We have transferred CD4 T cell clones of type 1 into infected SCID mice and found that this treatment had either no measurable effect or resulted only in temporary reduction in virus titer but never in recovery from infection (Scherle *et al.,* 1992, and unpublished observations).

The previous demonstration that infected SCID mice can be cured by treatment with anti-HA antibodies of IgG isotypes (Palladino *et al.,* 1995; Mozdzanowska *et al.,* 1997) is consistent with a crucial role of the T-dependent antibody response in the recovery of CD8-deficient mice. Recent studies showed that the antibody response plays an important role also in the recovery process of intact mice (Graham and Braciale, 1996; Gerhard *et al.,* 1997). Of note in this context is that antibody secreting cells (ASC) in draining lymph nodes arise between 4 and 6 days after infection (Justewicz *et al.,* 1995) which coincides with the time when virus titers are dropping. Also, we have previously identified a subpopulation of HA-specific B cells (termed C12) which is characterized by (1) use of a restricted set of unmutated V region genes, (2) rapid maturation to ASC within 5 days, and (3) early or immediate switch to IgG heavy chain isotypes (Kavaler *et al.,* 1990, 1991). This B cell population accounts for ~25% of the primary response on day 5 and members of this group have been shown to be highly effective therapeutically in SCID mice (Mozdzanowska *et al.,* 1997). A similar B cell population has recently been identified also in the response of mice to vesicular stomatitis virus (Kalinke *et al.,* 1996). The rapid kinetics of these responses is consistent with an important role of antibodies in the recovery process.

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REFERENCES

Baird, A., and Parker, D. C. (1996). Analysis of low zone tolerance induction in normal and B cell-deficient mice. *J. Immunol.* 157, 1833–1839.

Baumgarth, N., Brown, L., Jackson, D., and Kelsoe, A. (1994). Novel features of the respiratory tract T-cell response to influenza virus infection: Lung T cells increase expression of gamma interferon mRNA in vivo and maintain high levels of mRNA expression for interleukin-5 (IL-5) and IL-10. *J. Virol.* 68, 7575–7581.

Bender, B. S., Croghan, T., Zhang, L., and Small, P. A. (1992). Transgenic

mice lacking class I major histocompatibility complex-restricted T cells have delayed viral clearance and increased mortality after influenza virus challenge. *J. Exp. Med.* 175, 1143–1145.

Carding, S. R., Allen, W., McMickle, A., and Doherty, P. C. (1993). Activation of cytokine genes in T cells during primary and secondary murine influenza pneumonia. *J. Exp. Med.* 177, 475–482.

Chen, J., Troustine, M., Alt, F. W., Young, F., Kurahara, C., Loring, J. F., and Huszar, D. (1993). Immunoglobulin gene rearrangement in B cell deficient mice generated by targeted deletion of the J_H locus. *Int. Immunol.* 5, 647–656.

Constant, S., Schweitzer, N., West, J., Ranney, P., and Bottomly, K. (1995). B lymphocytes can be competent antigen-presenting cells for priming $CD4^+$ T cells to protein antigens in vivo. *J. Immunol.* 155, 3734–3741.

Doherty, P. C., Topham, D. J., Tripp, R. A., Cardin, R. D., Brooks, J. W., and Stevenson, P. G. (1997). Effector $CD4^+$ and $CD8^+$ T cell mechanisms in the control of respiratory virus infections. *Immunol. Rev.,* in press.

Doherty, P. C., Allan, W., Eichelberger, M., and Carding, S. R. (1992). Roles of $\alpha\beta$ and $\gamma\delta$ T cell subsets in viral immunity. *Ann. Rev. Immunol.* 10, 123–151.

Eichelberger, M., Allan, W., Zijlstra, M., Jaenisch, R., and Doherty, P. C. (1991). Clearance of influenza virus respiratory infection in mice lacking class I major histocompatibility complex-restricted $CD8^+$ T cells. *J. Exp. Med.* 174, 875–880.

Epstein, M. M., Di Rosa, F., Jankovic, D., Sher, A., and Matzinger, P. (1995). Successful T cell priming in B cell-deficient mice. *J. Exp. Med.* 182, 915–922.

Fitch, F. W., McKisic, M. D., Lancki, D. W., and Gajewski, T. F. (1993). Differential regulation of murine T lymphocyte subsets. *Ann. Rev. Immunol.* 11, 29–48.

Franco, M. A., and Greenburg, H. B. (1995). Role of B cells and cytotoxic T lymphocytes in clearance of and immunity to Rotavirus infection in mice. *J. Virol.* 69, 7800–7806.

Gerhard, W., Mozdzanowska, K., Furchner, M., Washko, G., and Maiese, K. (1997). Role of the B cell response in recovery of mice from primary influenza virus infection. *Immunol. Rev.,* in press.

Germain, R. N., Castellino, F., Han, R., Reis, C., Sousa, E., Romangnoli, P., Sadegh-Nasseri, S., and Zhong, G.-M. (1996). Processing and presentation of endocytically acquired protein antigens by MHC class II and class I molecules. *Immunol. Rev.* 151, 1–28.

Graham, M. B., and Braciale, T. J. (1996). Influenza virus clearance in B lymphocyte deficient mice. *In* "Options for the Control of Influenza III"(L. E. Brown, A. W. Hampson, and R. G. Webster, Eds.), pp. 166–169. Elsevier Science BV, New York.

Graham, M. B., Braciale, V. L., and Braciale, T. J. (1994). Influenza virus-specific $CD4^+$ T helper type 2 lymphocytes do not promote recovery from experimental virus infection. *J. Exp. Med.* 180, 1273–1282.

Haberman, A. M., Moller, C., McCreedy, D., and Gerhard, W. (1990). A large degree of functional diversity exists among helper T cells specific for the same antigenic site of influenza hemagglutinin. *J. Immunol.* 145, 3087–3094.

HayGlass, K. T., Naides, S. J., Scott, C. F., Benacerraf, B., and Sy, M.-S. (1986). T cell development in B cell-deficient mice. IV. The role of B cells as antigen-presenting cells in vivo. *J. Immunol.* 136, 823–829.

Hou, S., and Doherty, P. C. (1995). Clearance of Sendai virus by $CD8^+$ T cells requires direct targeting to virus-infected epithelium. *Eur. J. Immunol.* 25, 111–116.

Hou, S., Fishman, M., Gopal Murti, K., and Doherty, P. C. (1993). Divergence between cytotoxic effector function and tumor necrosis factor alpha production for inflammatory $CD4^+$ T cells from mice with Sendai virus pneumonia. *J. Virol.* 67, 6299–6302.

Janeway, C. A. S., Ron, Y., and Katz, M. E. (1987). The B cell is the initiating antigen-presenting cell in peripheral lymph nodes. *J. Immunol.* 138, 1051–1055.

Jonjic, S., Pavic, I., Polic, B., Crnkovic, I., Lucin, P., and Koszinowski, U. H. (1994). Antibodies are not essential for the resolution of primary cytomegalovirus infection but limit dissemination of recurrent virus. *J. Exp. Med.* 179, 1713–1717.

Justewicz, J. M., Doherty, P. C., and Webster, R. G. (1995). The B-cell response in lymphoid tissue of mice immunized with various antigenic forms of the influenza virus hemagglutinin. *J. Virol.* 69, 5414–5421.

Kalinke, U., Bucher, E. M., Ernst, B., Oxenius, A., Roost, H.-P., Geley, S., Kofler, R., Zinkernagel, R. M., and Hengartner, H. (1996). The role of somatic mutation in the generation of the protective humoral immune response against vesicular stomatitis virus. *Immunity* 5, 639–652.

Karasuyama, H., and Melchers, F. (1988). Establishment of mouse cell lines which constitutively secrete large quantities of interleukin 2, 3, 4 or 5, using modified cDNA expression vectors. *Eur. J. Immunol.* 18, 97–104.

Kavaler, J., Caton, A. J., Staudt, L. M., and Gerhard, W. (1991). A B cell population that dominates the primary response to influenza virus hemagglutinin does not participate in the memory response. *Eur. J. Immunol.* 21, 2687–2695.

Kavaler, J., Caton, A. J., Staudt, L. M., Schwartz, D., and Gerhard, W. (1990). A set of closely related antibodies dominates the primary antibody response to the antigenic site Cb of the A/PR/8/34 influenza virus hemagglutinin. *J. Immunol.* 145, 2312–2321.

Kitamura, D., Roes, J., Kuhn, R., and Rajewsky, K. (1991). A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin μ chain gene. *Nature (London)* 350, 423–426.

Kunding, T. M., Hengartner, H., and Zinkernagel, R. M. (1993). T cell-dependent IFN-γ exerts an antiviral effect in the central nervous system but not in peripheral solid organs. *J. Immunol.* 150, 2316–2321.

Kurt-Jones, E. A., Liano, D., HayGlass, K. A., Benacerraf, B., Sy, M.-S., and Abbas, A. K. (1988). Role of antigen-presenting B cells in T cell priming in vivo. Studies of B cell-deficient mice. *J. Immunol.* 140, 3773–3778.

Leung, K. N., and Ada, G. L. (1982). Different functions of subsets of effector T cells in murine influenza virus infection. *Cell. Immunol.* 67, 312–324.

Liang, S., Mozdzanowska, K., Palladino, G., and Gerhard, W. (1994). Heterosubtypic immunity to influenza type A virus in mice. Effector mechanisms and their longevity. *J. Immunol.* 152, 1653–1661.

Lightman, S., Cobbold, S., Waldmann, H., and Askonas, B. A. (1987). Do $L3T4^+$ T cells act as effector cells in protection against influenza virus infection. *Immunology* 62, 139–144.

Liu, Y., Wu, Y., Ramarathinam, L., Guo, Y., Huszar, D., Troustine, M., and Zhao, M. (1995). Gene-targeted B-deficient mice reveal a critical role for B cells in the CD4 T cell response. *Int. Immunol.* 7, 1353–1362.

Macaulay, A. E., DeKruyff, R. H., Goodnow, C., and Umetsu, D. T. (1997). Antigen-specific B cells preferentially induce $CD4^+$ T cells to produce IL-4. *J. Immunol.* 158, 4171–4179.

McNeal, M. M., Barone, K. S., Rae, M. N., and Ward, R. L. (1995). Effector functions of antibody and $CD8^+$ cells in resolution of rotavirus infection and protection against reinfection in mice. *Virology* 214, 387–397.

Moran, T. M., Isobe, H., Fernandez-Sesma, A., and Schulman, J. L. (1996). Interleukin-4 delayed virus clearance in influenza virus-infected mice. *J. Virol.* 70, 5230–5235.

Morrison, L. A., Lukacher, A. E., Braciale, V. L., Fan, D. P., and Braciale, T. J. (1986). Differences in antigen presentation to MHC class I- and class II-restricted influenza virus-specific cytolytic T lymphocyte clones. *J. Exp. Med.* 163, 903–921.

Mozdzanowska, K., Furchner, M., Washko, G., Mozdzanowski, J., and Gerhard, W. (1997). A pulmonary influenza virus infection in SCID mice can be cured by treatment with hemagglutinin-specific antibodies that display very low virus-neutralizing activity in vitro. *J. Virol.* 71, 4347–4355.

Palladino, G., Mozdzanowska, K., Washko, G., and Gerhard, W. (1995). Virus-neutralizing antibodies of immunoglobulin G (IgG) but not of

IgM or IgA isotypes can cure influenza virus pneumonia in SCID mice. *J. Virol.* 69, 2075–2081.

Phillips, J. A., Romball, C. G., Hobbs, M. V., Ernst, D. N., Shultz, L., and Weigle, W. (1996). $CD4^+$ T cell activation and tolerance induction in B cell knockout mice. *J. Exp. Med.* 183, 1339–1344.

Ramsay, A. J., Ruby, J., and Ranshaw, I. A. (1993). A case for cytokines as effector molecules in the resolution of virus infection. *Immunol. Today* 14, 155–157.

Ron, Y., and Sprent, J. (1987). T cell priming in vivo: A major role for B cells in presenting antigen to T cells in lymph nodes. *J. Immunol.* 138, 2848–2856.

Saoudi, A., Simmonds, S., Huitinga, I., and Mason, D. (1995). Prevention of experimental allergic encephalomyelitis in rats by targeting autoantigen to B cells: Evidence that the protective mechanism depends on changes in the cytokine response and migratory properties of the autoantigen-specific T cells. *J. Exp. Med.* 182, 335–344.

Sarawar, S. R., Sangster, M., Coffman, R. L., and Doherty, P. C. (1994). Administration of anti-IFN-γ antibody to β2-microglobulin-deficient mice delays influenza virus clearance but does not switch the response to a T helper cell 2 phenotype. *J. Immunol.* 153, 1246–1253.

Scherle, P. A., Palladino, G., and Gerhard, W. (1992). Mice can recover from pulmonary influenza virus infection in the absence of class I-restricted cytotoxic T cells. *J. Immunol.* 148, 212–217.

Steininger, B., and Sickel, E. (1991). Class II MHC molecules and monocytes/macrophages in the respiratory system of conventional, germfree and interferon-gamma-treated rats. *Immunobiology* 184, 295–320.

Stockinger, B., Zal, T., Zal, A., and Gray, D. (1996). B cells solicit their own help from T cells. *J. Exp. Med.* 183, 891–899.

Sunshine, G. H., Jimmo, B. L., Ianelli, C., and Jarvis, L. (1991). Strong priming of T cells adoptively transferred into scid mice. *J. Exp. Med.* 174, 1653–1656.

Taylor, P. M., Esquivel, F., and Askonas, B. A. (1990). Murine $CD4^+$ T cell clones vary in function in vitro and in influenza infection in vivo. *Int. Immunol.* 2, 323–328.

Taylor-Robinson, A. W., and Phillips, R. S. (1996). Reconstitution of B-cell-depleted mice with B cells restores Th2-type immune responses during *Plasmodium chabaudi chabaudi* infection. *Infect. Immun.* 64, 366–370.

Topham, D. J., and Doherty, P. C. (1997). Clearance of an influenza A virus by $CD4^+$ T cells is inefficient in the absence of B cells. *J. Virol.* (in press).

Topham, D. J., Tripp, R. A., Hamilton-Easton, A. M., Sarawar, S. R., and Doherty, P. C. (1996a). Quantitative analysis of the influenza virus-specific $CD4^+$ T cell memory in the absence of B cells and Ig. *J. Immunol.* 157, 2947–2952.

Topham, D. J., Tripp, R. A., Sarawar, S. R., Sangster, M. Y., and Doherty, P. C. (1996b). Immune $CD4^+$ T cells promote the clearance of influenza virus from major histocompatibility complex class II $-/-$ respiratory epithelium. *J. Virol.* 70, 1288–1291.

Vella, A. T., M. T. Scherer, M. T., Shultz, L., J. W. Kappler, J. W., and P. Marrack, P. (1996). B cells are not essential for peripheral T-cell tolerance. *Proc. Natl. Acad. Sci. USA* 93, 951–955.

Wells, M. A., Albrecht, P., and Ennis, F. A. (1981). Recovery from a viral respiratory infection. I. Influenza pneumonia in normal and T-deficient mice. *J. Immunol.* 126, 1036–1041.

Woegel, T., VanPel, A., De Plaen, E., Lurquin, C., Maryanski, J. L., and Boon, T. (1987). Immunogenic (tum^-) variants obtained by mutagenesis of mouse mastocytoma P815. *Immunogenetics* 26, 178–187.